# Forskolin induces circadian gene expression of *rPer1*, *rPer2* and *dbp* in mammalian rat-1 fibroblasts

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**Abstract Mammalian culture cells have the potential for periodicity, since high concentrations of serum can elicit the circadian expression of clock genes in rat-1 fibroblasts. However, the mechanism by which serum affects circadian gene expression remains unclear. In the present study, we incubated rat-1 cells with forskolin and successfully induced the rhythmic expression of *Per1*, *Per2* and *dbp*. In the initial step of the circadian gene expression, a marked transient induction of *Per1* was observed accompanied with CREB phosphorylation. Thus the present study strongly suggests that CREB activation through the cAMP/PKA pathway is involved in the generation of circadian rhythm in rat-1 cells**



*Key words:* Forskolin; CREB; Per1; Per2; Circadian rhythm

## 1. Introduction

All living organisms on earth have unique biological rhythms having periods of approximately 24 h long. They are called circadian rhythms and in mammals, the master circadian pacemaker controlling behavioral and hormonal circadian rhythmicity resides in the suprachiasmatic nucleus (SCN) located in the hypothalamus of the brain [1,2]. In fact, the putative clock genes such as *mPer1* [3,4] and *mPer2* [5–7] are all abundantly expressed in the SCN. Further molecular analyses of these genes suggest that the core oscillation mechanism common in most organisms is also approved in the mammalian circadian system [8]: the negative feedback loop for the *Per1* gene seems to work [9,10] like the *Drosophila per* gene where PER protein negatively regulates expression of its own gene [11]. However, precise in vivo analyses are hampered by the size (500 μm in diameter) and the localization of SCN, which is deep in the basal region of the brain.

The recent demonstration of clock gene expression in whole bodies [12] suggests that biological clocks are localized in many peripheral organs in mammals, as they are in *Drosophila* [13]. Furthermore, the finding of Balsalobre et al. [14], demonstrating the circadian expression of *Per1*, *Per2* and clock-related genes with immediate induction of *Per1* and *Per2* in cultured rat-1 fibroblasts after exposure to high concentrations of serum (serum shock), suggests clock oscillating activity at the cellular level. This also suggests the use of cell lines as tools with which to elucidate the mammalian mechanism of the molecular clock after specific stimulation. However, little is known of what 'serum shock' really is to initiate the rhythm in cultured cells.

It is well known that a cAMP-mediated mechanism is important for the phase-resetting of the circadian rhythm [15–17]. Forskolin elevates the cAMP level, and through the protein kinase A (PKA) signaling pathway, it enhances the phosphorylation and activation of $Ca^{2+}$/cAMP responsive element binding protein (CREB) [18,19]. In the present study, we investigate the effect of forskolin on circadian clock gene expression in rat-1 fibroblasts; we found that forskolin treatment elicited circadian gene expression of *rPer1*, *rPer2* and *dbp*, with acute induction of the *rPer1* gene and phosphorylation of CREB.

## 2. Materials and methods

### 2.1. Cell culture

Rat-1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 50 U/ml penicillin, and 0.05 mg/ml streptomycin at 37°C with 5% $CO_2$. For the experiments, $5\times10^5$ cells were plated on 6 cm dishes and cultured in DMEM containing 5% FBS for 4–5 days. Cells reach confluence after about 2 days under these conditions.

### 2.2. Forskolin and 50% horse serum treatment

At the point of 0 h, medium was changed to DMEM supplemented with forskolin (10 μM, final concentration; Nacalai Tesque, Kyoto) or DMEM supplemented with 50% horse serum (Gibco BRL), and 2 h after these media were replaced with serum-free DMEM supplemented with penicillin-streptomycin. At the indicated time, cultured cells were washed three times with ice-cold PBS and harvested in 1 ml of TRIzol reagent (Gibco BRL). These samples were frozen and stored at −70°C until the extraction of whole cell RNA. In the experiments of Fig. 3, forskolin- and 50% horse serum-supplemented media were not replaced by serum-free medium.

### 2.3. Northern blot analysis

Ten microgram of total RNA was electrophoresed in a 1.2% agarose gel containing 2% formaldehyde. RNAs were transferred to Biodyne Nylon Membrane (PALL BioSupport, New York) and hybridized with probes. A *rPer1* cDNA fragment (positions 736–1720 of *mPer1*) [4] and a *rPer2* cDNA fragment (positions 388–1898 of *mPer2*) [7] were PCR-amplified using the following oligonucleotides: 5′-CCATGGACATGTCTACT-3′ and 5′-ATGTCCCCTGGTCCTCT-3′ for *rPer1*, and 5′-ACCCCTCCACGAGCGGCTGCAGTA-3′, 5′-ACTGTCCAGGCAGCTGAT CTGCTG-3′ for *rPer2*. These fragments were then cloned into the pGEM-T Easy vector (Promega, Madison, WI) and the inserts digested by *Eco*RI were used as templates for the *rPer1* and *rPer2* probes. For *dbp*, the total coding region of mouse *dbp* (GenBank accession number U29762) was cloned by RT-PCR and ligated into the pCR2.1 TOPO vector (Invitrogen).

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G3PDH (Clontech) was used as a control. Probes were incubated overnight with membranes at 42°C, washed twice in 0.2×SSC/0.1% SDS at 60°C for 30 min, exposed to Imaging Plates and analyzed by BAS 5000 (Fuji Film, Tokyo).

### 2.4. Immunoblot

At the indicated time, rat-1 fibroblasts were washed three times with ice-cold PBS, and harvested in 0.3 ml of SDS Sample Buffer (125 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 1 mM PMSF, 50 mM NaF, 100 μM $NaVO_3$, 40 mM DTT). After these samples were boiled for 5 min, 20 μl of these samples were separated in 10% SDS-PAGE and transferred to PVDF membrane (Immunoblot-P membrane; Atto, Tokyo). As primary antibodies, anti-phospho-CREB (1:1000; NEB), and anti-CREB (1:1000; NEB) affinity-purified rabbit polyclonal antibodies were used. Horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2000; NEB) was used as a secondary antibody. Chemiluminescence was performed using Renaissance Western blot reagent plus (NEN, cat No. NEL105), and analyzed by LAS 1000 (Fuji Film).

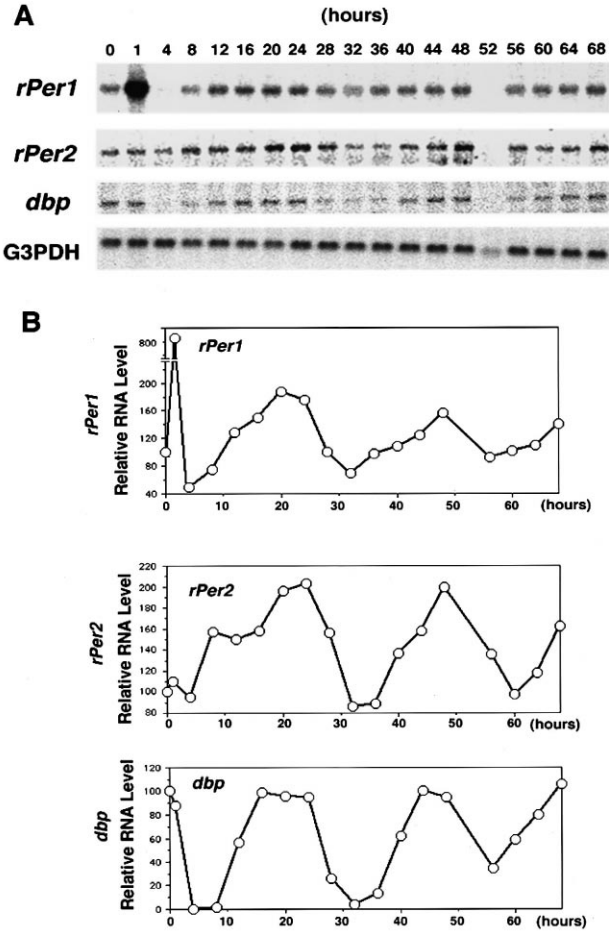


Fig. 1. Forskolin induced circadian gene expression of *rPer1*, *rPer2* and *dbp* mRNAs. A: Northern blot analysis for induction and subsequent circadian rhythmic expression of *rPer1, rPer2* and *dbp* mRNAs in forskolin-stimulated rat-1 fibroblasts. Culture and forskolin treatment of rat-1 cells were performed as described (see Section 2). Total RNAs were extracted at the indicated times and 10 μg of each total RNA was analyzed by Northern blotting (see Section 2) using *rPer1*, *rPer2* and *dbp* antisense probes as indicated on the left. G3PDH was used as control. B: The *rPer* positive signals obtained by Northern blot were quantified using a BAS 5000 Image Analyzer (Fuji Film). Robust circadian oscillations in gene expression were seen in *rPer1*, *rPer2* and *dbp*.

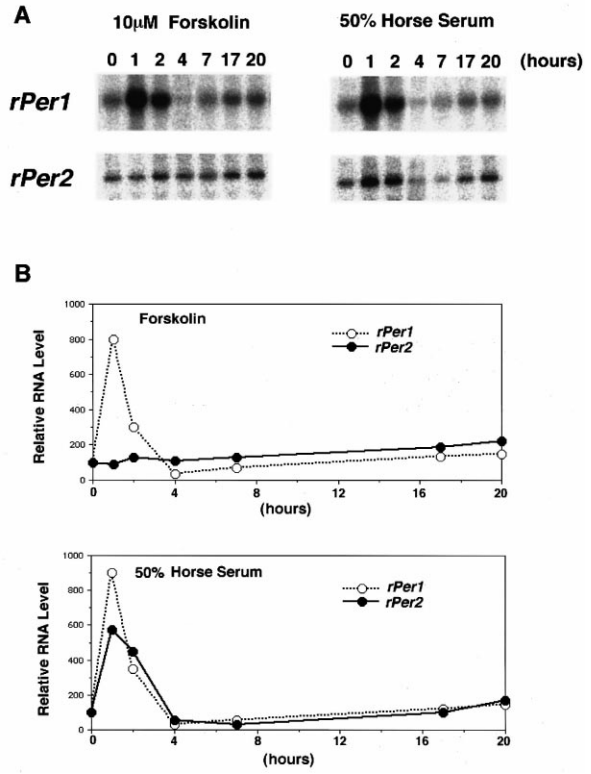


Fig. 2. Forskolin induced *rPer1* but not *rPer2* mRNA in rat-1 cells. Forskolin (10 μM final concentration) and 50% horse serum (serum shock) were administered to rat-1 fibroblast cultures in DMEM supplemented with 5% FBS. At 2 h after the treatment, medium was washed out and replaced with serum-free DMEM. A: Northern blot analysis was performed at indicated time points after these stimulations. Forskolin (upper panel) induced *rPer1* mRNA 1 h after stimulation, which was similar to the treatment with serum shock (lower panel). Although the serum shock strongly induced *rPer2* RNA, forskolin treatment shows neither rapid induction nor later suppression of *rPer2* mRNA. B: Quantification of signals obtained by the Northern blot in A by BAS 5000 Analyzer (Fuji Film). The signal levels at time = 0 were set at 100.

## 3. Results

### 3.1. Forskolin induced circadian gene expression of rPer1, rPer2 and dbp

Balsalobre et al. [14] reported that treatment with high concentrations of adult horse serum (serum shock) induced the circadian expression of *rPer1*, *rPer2*, *dbp* and some other genes in rat-1 fibroblasts. To investigate the involvement of cAMP on the circadian rhythmic expression of these genes, we applied forskolin (10 μM for 2 h) to the cultured rat-1 cells, and performed Northern blot analysis to examine the expression of *rPer1*, *rPer2* and *dbp* using whole cell RNA every 4 h for 3 days.

A brief exposure to forskolin induced rhythmic expression of all three genes for at least three circadian cycles, although control G3PDH gene did not show a circadian rhythm (Fig. 1A). After an initial acute increase, *rPer1* mRNA was repressed and began to show a rhythm of about 24 h period length consisting of peaks after 20–24 h and 48 h and troughs at 4 h, 32 h and 56 h. Levels *rPer2* mRNA remained low until 4 h, and then showed rhythms with peaks at 20–24 h and 48 h and with troughs at 32–36 h and 60 h. Levels of *dbp* mRNA decreases until 8 h, and then peaked at 16–24 h and 44–48 h

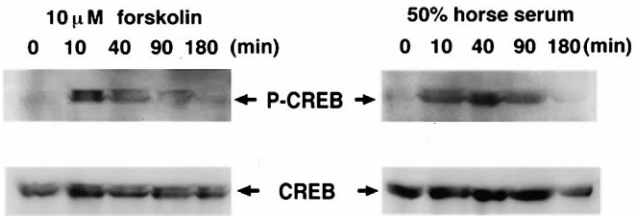


Fig. 3. Forskolin induced phosphorylation of CREB in rat-1 cells. Rat-1 fibroblasts cultured in 5% FBS containing DMEM for 4 days were stimulated with medium containing 10 μM forskolin (left panels) or 50% horse serum (right panels). Samples were harvested at the indicated times and analyzed by immunoblot. The upper panels show that both forskolin and serum shock induced the phosphorylation of CREB (P-CREB). The lower panels show total CREB protein using anti-CREB antibody.

with troughs at 32 h and 56 h. The peak and troughs of RNA accumulations were synchronous, and their peak/trough times were almost the same as those in serum shock (Fig. 1; [14] and our unpublished observation).

### 3.2. Forskolin strongly induced rPer1 mRNA but not rPer2 mRNA

Light induces a rapid induction of *Per1* mRNA that is closely correlated with phase-shifting of the clock [20]. Autonegative feedback suppression of products of the initial increase of *Per1* and *Per2* may likewise be important for the initiation of the serum-induced rhythmic expression [14]. Here, we examined the initial state of *rPer1* and *rPer2* mRNA in rat-1 fibroblasts after treatment with 10 μM of forskolin in detail, compared with those in serum shock (treatment with 50% horse serum). Forskolin rapidly induced *rPer1* mRNA 1 h after the treatment, followed by several hours repression with maximal suppression after 4 h (Fig. 2). The induction and the subsequent suppression of *rPer1* mRNA was also observed after the serum shock (Fig. 2). However, forskolin did not seem to affect *rPer2* mRNA expression (Fig. 2), in contrast to the acute induction and the subsequent repression of *rPer2* by serum shock.

### 3.3. Forskolin as well as serum shock induced the phosphorylation of CREB in rat-1 cells

In the mouse SCN, a brief exposure to light during subjective night not only induces *Per1* [20] but also accelerates the phosphorylation of CREB [21]. Since *Per1* is induced by forskolin in rat-1 cells in vitro, we performed immunoblot analysis using anti-phospho-CREB-specific antibody in rat-1 cells after exposure to forskolin or to serum shock.

Both forskolin and serum shock dramatically increased the phosphorylation of CREB, although the total amount of CREB was unchanged (Fig. 3). Forskolin treatment showed maximal phosphorylation after 10 min, which gradually decreased and returned to normal at 180 min. Serum shock also began the phosphorylation at 10 min and increased its level until 40 min, and returned to normal at 180 min.

## 4. Discussion

In the present study, we demonstrated that forskolin induces the circadian expression of putative clock oscillatory genes (*Per1* and *Per2*) and a clock-controlled gene (*dbp*) in rat-1 fibroblasts. Forskolin directly activates adenylate cyclase, which enhances the synthesis of native cAMP and the indirect activation of PKA. This pathway activates CREB by phosphorylation [19]. Thus the present study strongly suggests that cAMP/PKA pathways, which lead to activation of CREB, are involved in the generation of circadian rhythm in rat-1 cells.

Stimulating rat-1 cells with serum induces cell cycle progression with expression of several cell cycle-regulated genes [22]. However, as described by Balsalobre et al. [14], the generation time of rat-1 cells (15 h) is considerably shorter than the period length (~24 h) observed for serum shock-induced mRNA accumulation. As regards the stimulation with forskolin, cAMP acts as an antimitogenic signal in rat-1 fibroblasts [23], whereas serum stimulation progresses cell proliferation. Although cAMP and serum stimulation act in opposite directions for rat-1 cells, similar period lengths could be observed in forskolin-stimulated and serum-shocked experiments. Culturing cells in serum-free medium, which puts cells in the quiescent condition of cell growth, for 3 days after 2 h stimulation with forskolin and serum shock will also exclude the effect of cellular proliferation.

In this study, we demonstrated that forskolin rapidly induced and then suppressed *rPer1* RNA expression, results that were extremely similar to the response of *rPer1* after serum shock [14]. The immediate increase of *Per1* mRNA by exposure to forskolin has also been identified in cells from ovine pars tuberalis [24]. An autonegative feedback loop of clock oscillating genes seems to be formed in mammals as in *Drosophila*. Therefore, the acute induction of *Per1*, a putative oscillating gene in mammals, followed by the subsequent suppression, is likewise required to initiate the rhythm formation.

On the other hand, the *Per2* gene showed neither an immediate response nor later suppression after the forskolin treatment, both of which were evident after the serum shock. This suggests that there are different regulatory mechanisms between *rPer2* and *rPer1* gene expression, in addition to the common mechanism. Moreover, the initial lack of response to forskolin suggests that *rPer2* rhythm generation in the forskolin rat-1 system is a secondary phenomenon generated by the *rPer1* oscillation. Since both *Per1* and *Per2* may have clock oscillating activity, the synchronizing mechanism of these two genes needs further study.

In the present study, we demonstrated that phosphorylation of CREB is increased in both forskolin and serum shock treatments. Although serum shock will influence many intracellular pathways, we recently found that the serum stimulation rapidly increased the phosphorylation of p42/44 mitogen-activated protein kinases which activate CREB by phosphorylation (Yagita and Okamura, unpublished observation). CREB phosphorylation is believed to be the main pathway to reset the clock by a light pulse [21]. In this context, it is very likely that forskolin and serum shock can reset the clock in each of the cultured cells at irregular phase, to the new organized ensemble phase of cells via phosphorylation of CREB. Further works could use this cultural cell line to understand the molecular mechanism of mammalian clock generation.

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